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IN VITRO ASSESSMENT OF PLANT GROWTH PROMOTING POTENTIAL OF *KLEBSIELLA PNEUMONIAE, BACILLUS AMYLOLIQUEFACIENS* AND *BACILLUS SP.*

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ABSTRACT

Biosynthesis of Indole-3-acetic acid (IAA), phosphate solubilization and siderophore production plays most important role in the plant growth promotion. The present study aimed to investigate the effect of various concentrations (5µg/ml, 10µg/ml, 25 µg/ml and 40µg/ml) of zinc compounds like $ZnCl_2$, $ZnSO_4.7H_2O$, ZnO and CH_3COOZn on the IAA production by six rhizobacterial strains namely MR-M1, MR-AI, MR-SP, RR-R2, WR-W2 and MR-Z1 under *in vitro* conditions. To study siderophore biosynthetic potentials on the basis of orange/yellow halo zone formation, isolates were grown onto JNFb and basal medium containing 1µM of $FeCl_3$. JNFb medium was observed as the most suitable medium for siderophore production. More than two fold stimulation of IAA production was observed in presence of zinc compounds. The highest level of IAA was recorded in presence of $ZnSO_4$ as compared to other zinc compounds. Strain *Bacillus subtilis* WR-W2 followed by strain *Klebsiella pneumonia*e MR-M1 was observed as the most efficient strain in terms of their capability to produce siderophore. However, strain *Klebsiella pneumonia*e MR-M1 was observed as the best IAA producer and phosphate solubilizer as compared to other *Bacillus sp*. MR-AI, MR-SP, RR-R2, WR-W2 and MR-Z1. Altogether, *Klebsiella sp*. and *Bacillus sp*. reveal multiple beneficial properties for plant growth promotion that could be further applied as bioinoculums in different formulation in crop fields for sustainable agriculture.

KEYWORDS: Plant Growth Promoting Rhizobacteria, Siderophore Production, Zinc

INTRODUCTION

PGPR help plant growth by a combination of physiological attributes such as asymbiotic N_2 fixation, production of phytohormones like IAA, by solubilizing insoluble mineral phosphate and by siderophore production (Neilands, 1981, Rodriguez and Fraga, 1999; Ahemad and Kibret 2013). Reports reveals diverse bacterial genera such as including *Bacillus* and *Klebsiella* frequently colonize the important cereal crops and promote plant growth under *in vitro* as well as *in vivo* (Kloepper *et al*.,2004; Saharan and Nehra 2011, Ahemad and Kibret 2013). IAA and siderophore are a secondary metabolite generally produced in early stationary phase of growth of bacteria. L-tryptophan serves as physiological precursor for biosynthesis of IAA in plants and in microbes (Frankenberger and Arshad 1995).

Iron is the 4th abundantly available element in the earth crust (Archibald 1983). Although, iron is present in 1-6% but is unavailable to microbes as well as plants, due to its insoluble iron oxyhydroxide polymers under aerobic conditions at biological pH. Siderophore, are low molecular weight, iron chelating ligands, synthesized under iron-limited conditions (Neilands, 1981). Siderophore binds iron with an extremely high affinity and is specifically recognized by a corresponding outer membrane receptor protein, which in turn actively transported the complex into the periplasm of the cell. Many bacteria are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores (Neilands, 1981). Microbial siderophores may stimulate plant growth directly by increasing the

availability of iron in the soil surrounding the roots (Kloepper *et al.,*1980). Reports suggest that plants may utilize siderophores synthesized by soil microorganisms (Marschner and Römheld, 1994). Enterobactin is produced by a number of bacteria including *E. coli* and is the classic example of a catechol-type siderophore (Pollack *et al.,* 1970). Catecholate or phenolate type of siderophore bind, Fe^{3+} using adjacent hydroxyl groups of catechol rings Enterobactin production has been demonstrated in some N2-fixing bacteria, including *Klebsiella pneumoniae* (Höfte 1993). Moreover, hydroxamate-type siderophores such as aerobactin produced by *E. coli*, ferrichrome utilized by various bacterial species (Höfte, 1993). On the other hand, PGPR convert insoluble phosphorus to an accessible form, like orthophosphate, is an important trait for a PGPR for increasing plant yields. Phosphorous (P) is next to N2 which limits plant growth. The concentration of soluble P in soil is usually at levels of 1 ppm or less 10 M $H_2PO_4^-$. The cell might take up various P forms but the greatest part is absorbed in the forms of HPO_4^{2-} or H_2PO^{4-} (Rodriguez and Fraga, 1999). In fact, a large portion of inorganic phosphates applied to soil as fertilizer are rapidly immobilized after application and become unavailable to plants. The production of organic acids such as gluconic acid by soil microorganisms is the principal mechanism for mineral phosphate solubilization (Khan *et al.,* 2007).

PGPR are commonly used as inoculants for improving the growth and yield of agricultural crops, however screening for the selection of efficient PGPR strains is very critical. The study focused on the screening of efficient PGPR strains on the basis of their IAA production, phosphate solubilization and siderophore production under *in vitro* condition. An individual strain having combination of all the above beneficial characters are of rare occurrence.

MATERIALS AND METHODS

Source, Culture Media and Incubation Condition

To isolate most efficient siderophore producing PGPR, soil samples were collected from rice, (RR-R2) wheat (WR-W2) and maize (MR-M1, MR-SP, MR-MZ and MR-AI) agricultural field of Banaras Hindu University, Varanasi, Uttar Pradesh, India. One kg rhizospheric soil sample were brought to the laboratory in the sterile polythene bags. Isolation of bacteria from rhizospheric soil was performed, following the standard microbiological methods of Barraquio *et al.,* (2000). One gram of rhizospheric soil of each sample were suspended in 10ml sterilized double distilled water (DDW) separately and serially diluted in 50ml Borosil glass tubes, up to 10 dilutions with three replicates. Siderophore test was performed in iron free basal and JNFb liquid and Agar-agar added medium (Sambrook and Russel 2001). The soil suspension obtained was used to pour onto Luria Bertani (L.B.) medium containing Yeast extract 5.0 (g/l), NaCl 5.0(g/l),tryptone 10 (g/l) at Ph 7.0 (Sambrook and Russel, 2001) and purified colony was sub-cultured onto basal medium the ingredients and concentrations are K_2HPO_4 5.0g/l, KH_2PO_4 2.0 g/l, $MgSO_47H_2O$ 0.5 g/l, $CaCl_2.2H_2O$ 0.02 g/l, $FeSO_4.7H_2O$ 0.001 g/l, Peptone1.0 g/l, Dextrose1.0 g/l (Sambrook and Russell, 2001). 10µl inoculums were taken from 10^{-1} to 10^{-10} dilutions and plated onto both the solid culture plates in duplicate set, incubated at 32^0C for 24h. Pure culture was isolated from rice, wheat and maize soil samples by streaking three to four times repeated sub-culture on the fresh slightly modified Johanna Nitrogen fixing bacteria (JNFb) agar-agar medium containing Malic acid 5.0g/l, K_2HPO_4 0.60 g/l, KH_2PO_4 1.80g/l, $MgSO_47H_2O$ 0.20g/l, NaCl 0.10g/l, $CaCl_2.2H_2O$ 0.20Gg/l, $Na_2MoO_4.2H_2O$ 0.002g/l, KOH 4.5g/l,Fe.-EDTA (1.4%) 4.0ml and NH_4Cl, 2.5mM at pH 5.8 (Dobereiner *et al.,* 1995). pH was maintained at 5.8 with the help of KOH and HCl by cyberscan ph ion 510 bench pH/ion/mv emter (Eutech instruments Pvt Ltd, Singapore). Six most efficient IAA producing rhizospheric bacteria were selected for plant growth promoting activity study namely MR-M1, MR-SP, MR-Z1, MR-AI, WR-W2 and RR-R2. All the selected strains were repeatedly sub-cultured and maintained onto JNFb solid plate.

IAA Production

To check IAA production by the six strains JNFb medium additionally supplemented with L-tryptophan (Loba Co.) (200µg/ml) were used. Various concentrations as 0, 5, 10, 25 and 40µg/ml of zinc compounds were added in the culture tube to six strains separately in JNFb medium containing 1µM of $FeCl_3$. Control set devoid of any zinc compound containing tryptophan only. $1x10^6$ concentrations of inoculums was transferred into 10ml of freshly prepared medium, incubated in 50 ml Borosil glass tubes with three replicates at 30^0C-32^0C on a rotary shaker for 92-96h. The optimum time for maximum IAA production growth was measured turbidimetrically by an EEL (Evans Electroselenium Ltd, Engaland) calorimeter at 530 nm and expressed in EEL units following calorimetric estimation of IAA by the method developed by Gordon and Weber (1952). 2.0 ml of IAA reagent containing 1.0ml 0.5 M $FeCl_3$ and 50 ml 35% $HClO_4$ was mixed to 1ml of cell free supernatant and optical density was taken at 530nm after 25min.

Phosphate Solubilization

To check Phosphate solubilizing all the strains were grown separately onto National Botanical Research Institute's phosphate (NBRIP) medium (Nautiyal *et al.*,1999) containing Glucose 10.0g/l, Ca_3PO_4 5g/l, $MgSO_4.7H_2O$ 1g/l, KCl 0.2g/l, NaCl 1g/l, NH_4Cl 5 g/l (ph 7.0). In order to estimate phosphatase activity, all the six strains were grown in Nautiyal's liquid medium and incubated in rotary shaker for 96h at 32^0C. 1.0 ml of the cell free supernatants of the grown culture was used for phosphatase estimation by the method developed by (Rodriguez and Fraga, 1999). Free P released in the medium as a result of phosphate solubilization by the isolates was estimated as per the method of Marinetti (1962). Containing, 1-part of reagent A (10% ascorbic acid) and 6-part freshly prepared reagent B (0.42% $[(NH4)_2MoO_4]$ in 1 N H_2SO_4). 1.0 ml of cell free supernatant of above culture were added with 3.0 ml of phosphorus test reagent and incubated for 1h. Appearance of blue color indicated production of dissolved phosphorus, which ensured production of gluconic acid, released by the strains to make inaccessible tricalcium phosphate to plant accessible form. Absorbance was recorded at 660 nm using UV spectrophotometer. Estimation of P solubilized was performed using a separately prepared standard of different concentration of K_2HPO_4. Control was maintained without tricalcium phosphate.

Quantitative Estimation of Siderophore Production

Qualitative detection of siderophore production was carried out by a highly sensitive chromogenic assay method, on chemically defined casamino acid (CAA) and chrome azurol S added (CAS, Hi Media, India) Agar-agar medium, as described by Neilands (1981) in completely iron free conditions. It contains casamino acid 5g/l, K_2HPO_4 1.18g/l and $M_GSO_4.7H_2O$ 0.25 g/l (pH 6.8). To obtain the iron decreased medium, chemically synthetic iron chelating agent ethylene diamine dihydroxyphenylacetic acid (EDDHA) was supplemented in liquid and solid medium. Based on their affinity for iron (III), and its effectiveness is therefore independent of their chemical structure. Agar plates were supplemented with 100 mM 2,29-dipyridyl in addition to CAS. One colony was used to inoculate blue/green agar CAS plates. Freshly prepared CAA medium mixed with JNFb and basal medium separately and final volume was maintained. Using the chrome azurol S (CAS) assay, when it is grown in a low iron conditions, chemically defined medium (CDM) develop a system suitable for better iron uptake under iron-stressed conditions. To prevent iron contamination, utmost care was taken by washing all the glassware's with EDTA followed by 1N HCl and with sterile MQW thrice. After 48 h of growth in above medium at 32^0C plates were observed. Since, siderophore possess strong affinity for iron (III), hence chelate iron from the dye. Resultantly, the color turns from blue to orange/yellow.

Preparation of CAS Agar Plates

To prepare 1L of CAS agar medium, 60.5 mg CAS (Chrome azurol S) was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mMHCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA (hexadecyltrimethylammonium) bromide dissolved in 40ml water. But in place of HDTMA, we have used N-cetyl-N, N, N-trimethyl ammonium bromide (C-TAB) in same concentration. The resultant dark blue liquid was autoclaved and mixed with 960 ml of sterilized CAA medium and is poured into the petriplates. Each plate received 25 ml of medium. Agar plates were supplemented with 100 mM 2, 29-dipyridyl in addition to CAS. One colony was used to inoculate blue agar CAS plates. Using the chrome azurol S (CAS) assay, when it is grown in a low iron conditions, chemically defined medium develop a system suitable for better iron uptake under iron-stressed conditions. Qualitative and quantitative estimation was performed after 96h at 32^0C.Colony diameter (CD) and halo zone diameter (HZD) was recorded.

Detection of Siderophore

Estimation of siderophore production was performed by universal CAS method of Schwyn and Neiland (1987)**.** 0.5 ml of cell free culture was added with 0.5 ml of CAS indicator reagent, incubated for 30 min. Appearance of pink color development was observed, which indicate siderophore positive and absorbance was recorded at 630 nm. The concentration of siderophore produced by the strains was estimated in terms of iron-binding equivalents using 2, 3-dihydroxybenzoic acids used as the standards and expressed as µg/ml. one control was separately prepared containing 1 µm $FeCl_3$.

Preparation of CAS Reagent

6 ml volume of 10 mM C-TAB in place of HDTMA solution was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron (III) solution (1mM $FeCl_3.6H_2O$ in 10mM HCl) and 7.5ml of 2mM aqueous CAS solution was slowly added under stirring. 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M HCl was carefully added to this buffer solution was rinsed into the volumetric flask, which was then filled with water to afford 100 ml CAS assay solution. The solution was stored in dark, preferable in polythene bottle.

Genomic DNA Isolation and Polymerase Chain Reaction of 16S rDNA

Whole cell genomic DNA was extracted following the standard protocol of Sambrook *et al.*, (1989). PCR amplification of 16S rDNA was performed following the method of Eckert *et al.*, (2001) with some modification using universal primer in a final volume of 50 µl. The PCR reaction mix included; 1.5U of Taq DNA polymerase (Banglore Genei, India), 1X PCR assay buffer, 25mM MgCl2, 20pmol each forward and reverse primers (Integrated DNA Technologies, Inc, CA, USA), each dNTPs:200µM (Banglore Genei, India) template DNA:50ng. Primer pair was forward 5'-AGA GTT TGA TYM TGG CTC AG-3' and reverse 5`-CTA CGG CTA CCT TGT TAC GA-3`). Amplification was performed in PTC-100 Thermal Cycler (MJ Research, Inc, Walthon, MA, USA), using initial denaturation at 94^0C for 30 sec, annealing at 57^0C for 1min, elongation at 72^0C and final extension at 72^0C for 5 min. and finally storage at 4^0C. 5µl of amplified reaction mixture was analyzed by agarose (2 % w/v) gel electrophoresis in TAE buffer (40 mM Tris, 1mM EDTA, pH -8.0). After run at 50 V for 3h, the gel was stained with ethidium bromide (0.5µg/ml) and photograph was taken in Gel-documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

DNA Sequencing

The 16S rDNA insert was sequenced by the dideoxy- chain termination method using an automated DNA sequencer (ABI Prism; Model 3100). To identify on genus level, PCR amplification and partial sequencing of 1.5Kb gene sequence and 400bp of hypervariable region between V2-V3 regions of 16S rRNA of the six strains were carried out. On the basis of highest percent similarity sequences obtained were searched through online available Basic Local Alignment Search tool (BLAST) tool of National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were submitted to the NCBI gene bank on http://www.ncbi.nlm.nih.gov.

Molecular Identification

Nucleotide sequence accession numbers of 16S rRNA gene sequences of the six strains are MR-AI identified as *Bacillus amyloliquefaciens* [FJ222551]. Strain MR-M1 revealed as *Klebsiella pneumonia*, [FJ222552]. Strain WR-W2 identified as *Bacillus subtilis* [FJ222553]. Strain MR-Z1 identified as *Bacillus subtilis* [FJ269243]. Strain RR-2 identified as *Bacillus subtilis* [EU327502]; Strain MR-SP identified as *Bacillus subtilis* [EU327504].

STATISTICAL ANALYSIS

Values were expressed as means of ± SD for triplicate samples. Differences were considered to be significant at the $P<0.05$ level.

RESULTS AND DISCUSSIONS

Here, we demonstrated that formation of clear visible orange/yellow halo zones around the periphery of colonies confirms production of siderophore after 96h of growth. These strains were found to produce maximum siderophore quantity during the stationary phase of culture growth (Sharma and Johri, 2003). Moreover, the level of siderophore production was compared in the two medium. Strain *Bacillus subtilis* WR-W2 followed by *Klebsiella pneumonia* MR-M1 was observed as the most efficient siderophore producer on the basis of JNFb agar agar plate assay (figure 1 and table 1.) and basal agar agar medium (figure 2. and table 2.). However, strain MR-SP was observed as the poor siderophore producer. The effect of chelation on siderophore production demonstrated maximal increase of siderophore halo zone diameter in WR-W2. Further increase in Fe (III) concentration caused a linear decrease to the siderophore production. The level of siderophore production was compared in the two medium. The siderophore production was greater in JNFb media. Studies reveal that the synthesis of siderophore depends on the chemical nature of the organic carbon and energy source (Mehri *et al.*, 2012). Although, the different media usually have varied levels of iron contamination, Sharma and Johri (2003) suggested that synthetic media are in all cases better than the complex medium for siderophore production. Complete inhibition of siderophore biosynthesis at concentration>20mM was reported by Carrillo-Castaneda *et al.*, (2005). Reports suggests *Bacillus subtilis*, secretes a catecholate (Ito and Neiland, 1958) and diazotrophic *Klebsiella pneumoniae* secrete enterobactin type of siderophore (Höfte 1993).

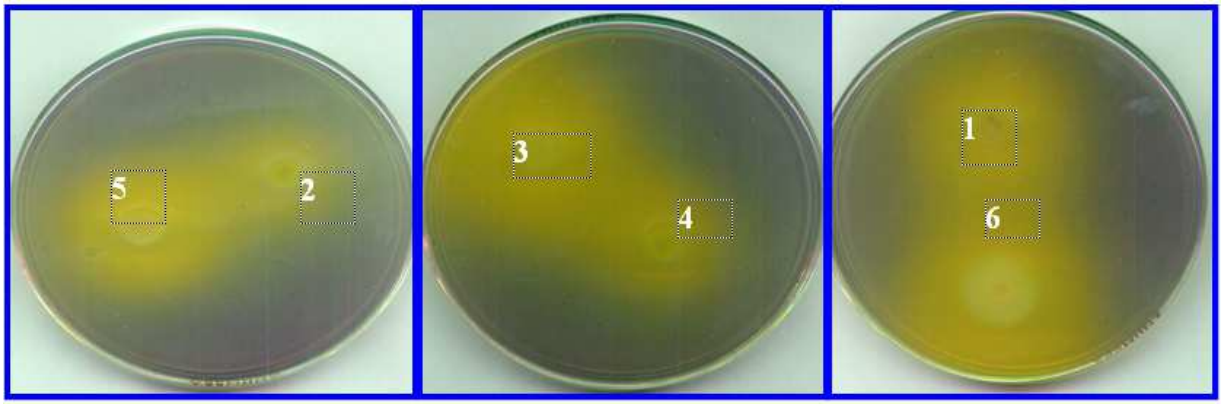

Figure 1: Siderophore Production in JNFb Medium, where 1. MR-M1, 2. MR-SP, 3.RR- R2, 4MR-MZ, 5.MR-AI, 6.WR-W2

Table 1: Determination of Colony Diameter and Halo Zone Diameter on the Basis of Figure 1

Strains	Colony Diameter (mm)	Halo Zone Diameter (mm)
MR-M1	1.10 ± 0.1	3.80 ±0.2
MR-SP	0.60 ± 0.2	0.90 ± 0.1
RR-R2	1.20 ± 0.1	3.20 ± 0.3
MR-Z1	1.10 ± 0.3	2.80 ± 0.2
MR-AI	1.00 ± 0.1	2.30 ± 0.4
WR-W2	1.90 ± 0.2	4.80 ± 0.5

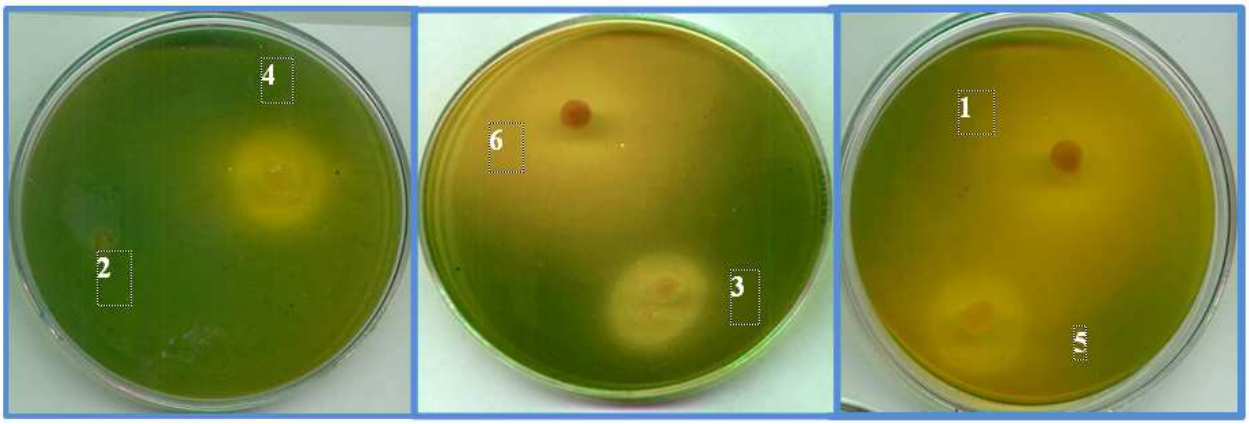

Figure 2: Siderophore Production in Basal Medium, Where 1.MR-M1, 2. MR-SP, 3.RR-R2, 4.MR-MZ, 5.MR-AI, 6.WR-W2

Table 2: Determination of Colony Diameter and Halo Zone Diameter on the Basis of Figure 2

Isolate	Colony Diameter (mm)	Halo Zone Diameter (mm)
MR-M1	2.50 ±0.4	4.80±0.2
MR-SP	0.30 ± 0.3	0.50±0.4
RR-R2	1.50 ± 0.4	2.55±0.2
MR-Z1	1.30 ± 0.3	2.00±0.3
MR-AI	2.70 ± 0.4	3.00±0.2
WR-W2	2.60 ± 0.3	5.50±0.2

Over the last decade, a significant research interest has been generated around PGPR, because of its phytostimulatory potentials. However, hardly any study has been reported to date regarding effect of zinc compounds on the siderophore production in the presently studied rhizobacteria. More than two fold increase in IAA production level was observed in presence of zinc compounds like ZnSO4 (figure 3.), ZnCl2 (figure 4.), ZnO (figure 5.) and CH3COOZn (figure 6.) as compared to control (without zinc compounds) (figure 7.). The media was supplement with $MgCl_2$. $MgSO_4.7H_2O$ to negate the role of chloride or sulphate ion. Diverse levels of IAA production were observed. Strain *Klebsiella pneumoniae* MR-M1 was observed as the most efficient IAA producer followed by *Bacillus subtilis* WR-W2. Effect of zinc compound on IAA production in *Klebsiella sp*. and *Bacillus sp*. has been well documented (Vivas *et al.*,2006; Bhatt and Vyas. 2014)). Complete inhibition of bacterial growth was observed at 50µg/ml of zinc.

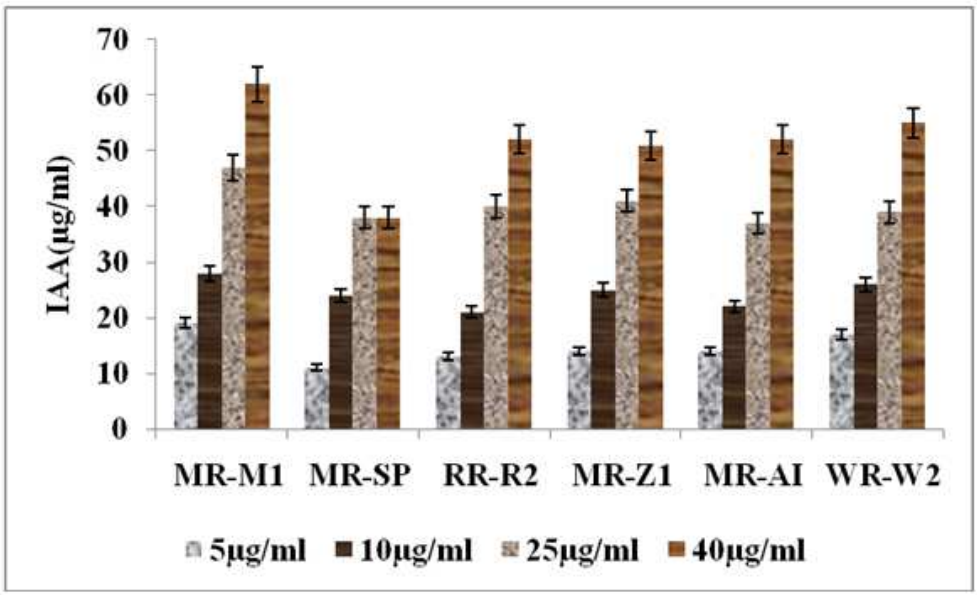

Figure 3: IAA Production in Presence of $ZnSO_4$

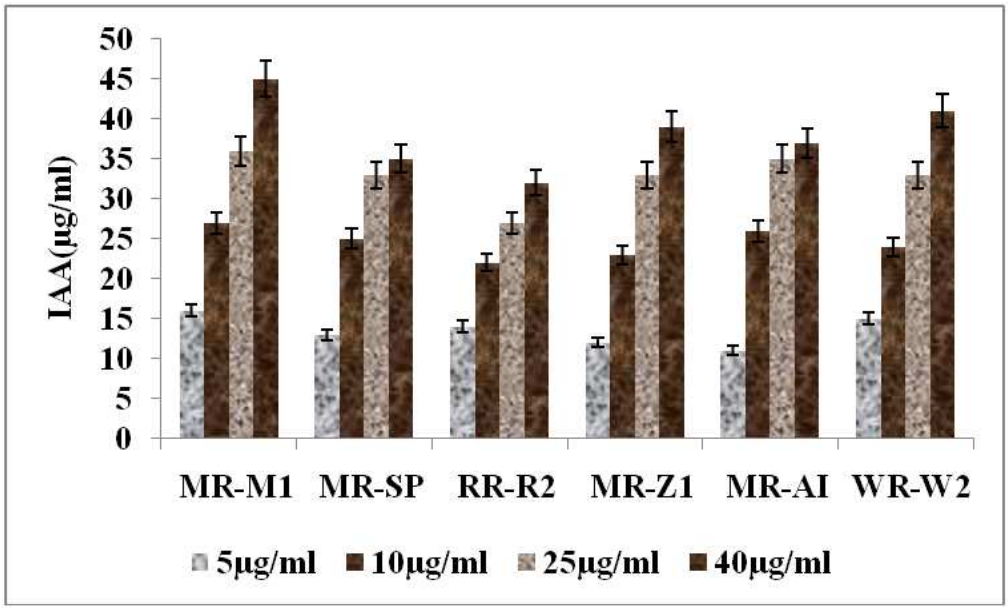

Figure 4: IAA Production in Presence of $ZnCl_2$

Phosphate solubilization potential of various PGPR has been well studied in *Bacillus spp*. (Rodriguez and Fraga.1999) and *Klebsiella pneumoniae* (Meulenberg *et al.*, 1990). Inorganic phosphate solubilization involves production of various organic acids like citric, gluconic and oxalic acids (Rodriguez and Fraga.1999). Here, we demonstrated the highest amount of phosphate solubilization was observed by strain *Klebsiella pneumonia*e MR-M1 followed by *Bacillus subtilis* WR-W2 (figure 8.). Result has been correlated with previous reports suggest generation of acidity and proton exchange mechanism are the plays key role in phosphate solubilization (Halder *et al.*, 1991). To our knowledge this is the first report demonstrated that strain *Klebsiella pneumoniae i*s more efficient in terms of IAA and phosphate solubilization capability then strains of *Bacillus* sp. under similar environmental and physical conditions.

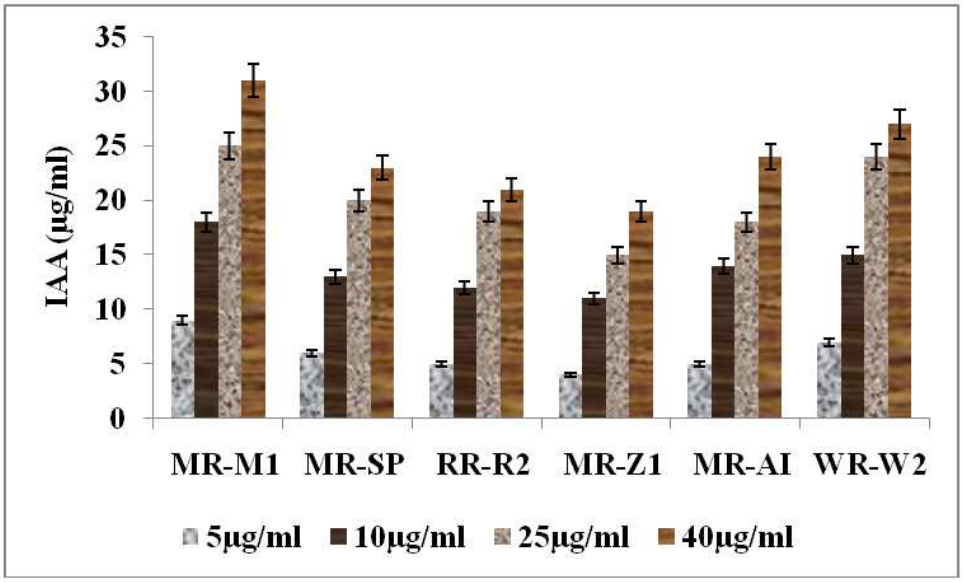

Figure 5: IAA Production in Presence of CH_3COOZn

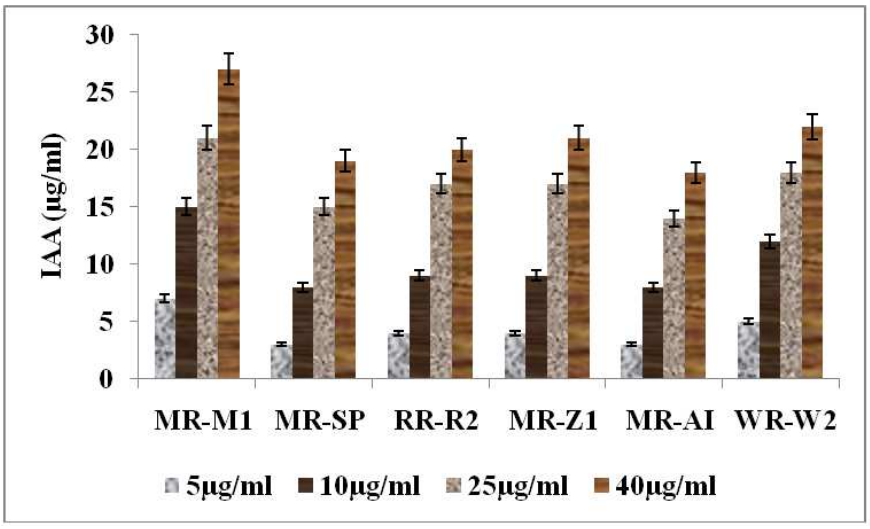

Figure 6: IAA Production in Presence of ZnO

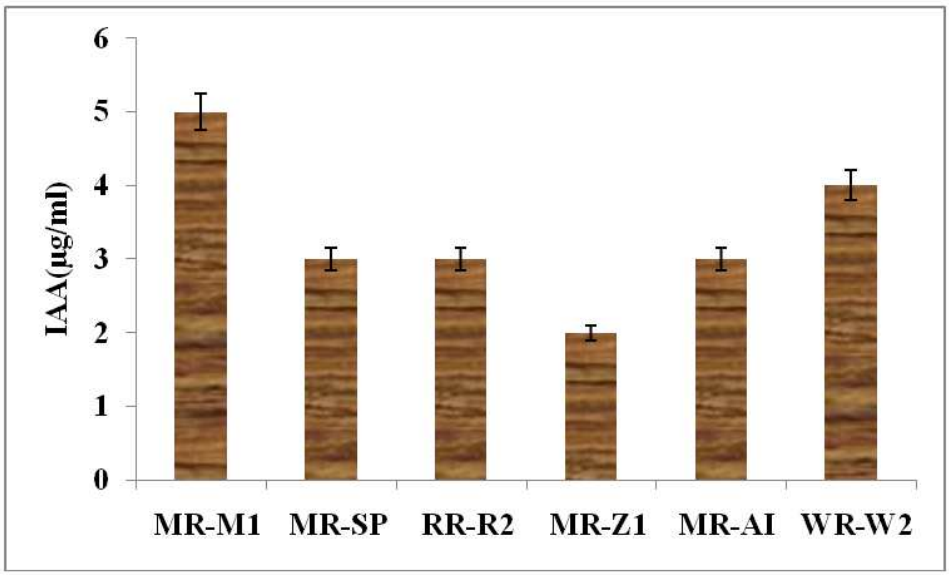

Figure 7: Quantitative Estimation of IAA Production in Presence of L-Tryptophan (100 µg/Ml) Without Zinc Compounds

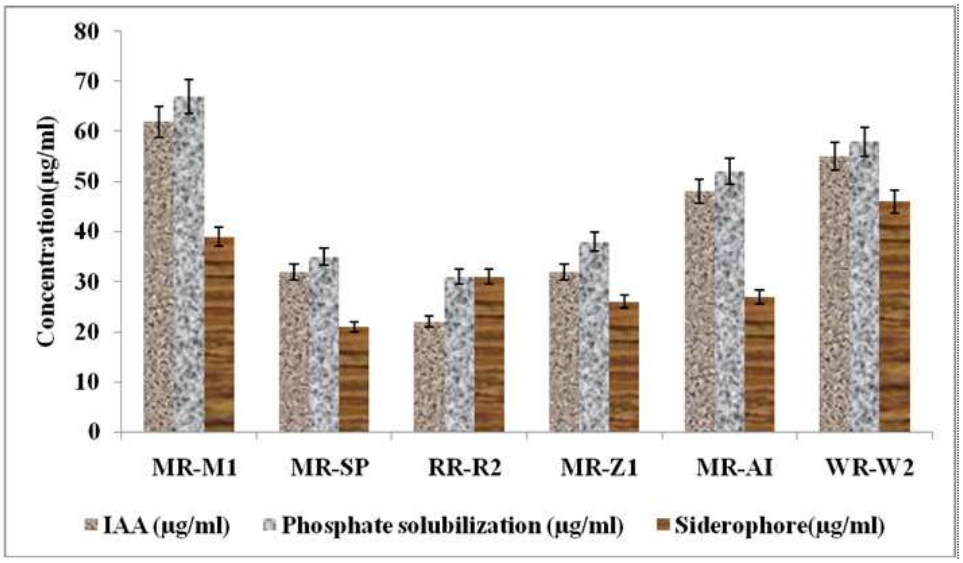

Figure 8: IAA Production in Presence of L-Tryptophan (200 µg/Ml), Phosphate Solubilization in Presence of Tri Calcium Phosphate (5.0g/L) and Siderophore Production in Presence of Fecl3 (1 µm)

CONCLUSIONS

In conclusion, our work reveals that zinc could significantly stimulate IAA production in rhizobacterial strains possess several beneficial characteristics like phosphate solubilisation and siderophore production. To our knowledge this is the first report showing Strain *Klebsiella pneumoniae* MR-M1 are more efficient in terms of IAA production and phosphate solubilization then five strains of *Bacillus sp*. MR-AI, MR-SP, RR-2, WR-W2 and MR-Z1 under *in vitro* conditions.

REFERENCES

1. Ahemad M, Kibret K (2013)**.** Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J. King Saud Univ. Sci. 26: 1-20**.**

2. Archibald F (1983). *Lactobacilhs plantarzrm*, an organism not requiring iron. FEMS Microbiol. Lett. 1: 29-32.

3. Barraquio W.L., Segubre E.M., Gonzalez M.S., Verma S.C., James E.K., Ladha J.K. and TripathiA.K. 2000. Diazotrophic enterobacteria: what is their role in rhizosphere of rice? In: J.K. Ladha and P.M. Reddy, Editors. The Quest for Nitrogen Fixation in Rice, Los Banos, Philippines. pp. 93- 118.

4. Bhatt P. B, Vyas B. R. M. (2014) Screening and Characterization of Plant Growth and Health Promoting Rhizobacteria Int.J.Curr.Microbiol.App.Sci 3(6): 139-155

5. Carrillo-Castaneda G, Munoz J. J, Peralta- Videa J. R (2005). A spectrophotometric method to determine the siderophore production by strains of fluorescent *Pseudomonas* in the presence of copper and iron. Microchem. J., 81: 35-40.

6. Dobereiner J. (1995). Isolation and identification of aerobic N2-fixing bacteria from soil and plants. In: Methods in Applied Soil Microbiology and Biochemistry, (Alef and P. Nannipieri Eds.). Academic Press, London, U.K. pp. 134-141.

7. Eckert B, Weber O.B, Kirchhof G, Halbritter A, Stoffels M. Hartmann A. (2001). *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass *Miscanthus*. Int.J. Syste. Evo. Microbiol. 51: 17-26.

8. Frankenberger JRWT, Arshad M (1995). Phytohormones in soils. Microbial Production and Function, Marcel Dekker, Inc. New York.pp. 5-40

9. Gordon SA, Weber RP (1951). Colorimeteric estimation of indole acetic acid. *Plant Physiol*. 26: 192-195.

10. Halder A.K., Misra A.K., Chakrabarty P.K. (1991) Solubilization of inorganic phosphates by Bradyrhizobium, Ind. J. Exp. Biol . 29, 28-31.

11. Höfte M, Buysens S, Koedam N, Cornelis P. (1993). Zinc affects siderophore-mediated high affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa*7NSK2. Biometals. 6(2):85-91.

12. Khan M.S., Zaidi A, Wani P.A., (2007). Role of phosphate-solubilizing microorganisms in sustainable agriculture - A review Agron. Sustain. Dev. 27 29-43.

13. Kloepper J.W, Ryu C.M, Zhang S (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* sp. Phytopathol. 94: 1259-1266.

14. Kloepper J.W, Leong J, Teintze M, Schroth M.N (1980). Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature. 286: 885-886.

15. Marschner H, Römheld V (1994) Strategies of plants for acquisition of iron. Plant and Soil. 165(2): 261-274.

16. Mehri I, Khessairi A, Turki Y, Saidi N, Daly I, Meyer J M, Hassen A (2012). Effect of Dose-Response of Zinc and Manganese on Siderophores Production. Am. J.Env. Sci. 8 (2): 143-151.

17. Marinetti GV (1962) Chromatographic separation, identification and analysis of phosphatides. J Lipid Res 3:1-20.

18. Meulenberg, J. J. M., E. Sellink, W. A. M. Loenen, N. H. Riegman, M. van Kleef, and P. W. Postma**.** 1990. Cloning of *Klebsiella pneumoniae pqq* genes and PQQ biosynthesis in *Escherichia coli*. FEMS Microbiol. Lett. 71:337-344.

19. Nautiyal C. S. (1999), An efficient microbiological growth medium for screening phosphate solubilising microorganisms. FEMS Microbiol. Lett. 170: 265-270.

20. Neilands J.B. (1981). Iron absorption and transport in microorganisms. Annual Rev. Nutr. 1: 27- 46.

21. Pollack J.R.,Neilands J.B (1970) Enterobactin, an iron transport compound from *Salmonella typhimurium* Biochem. Biophy. Res. Commun. 38(5):989-992.

22. Saharan BS, Nehra V (2011). Plant Growth Promoting Rhizobacteria: A Critical Review Life Sci. Med. Res. 21:1-30.

23. Sambrook J, Russel DW (2001). *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

24. Sambrook J, Fritsch E.F, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring harbor, New York, USA.

25. Schwyn B, Neilands J.B (1987). Universal chemical assay for the detection and determination of siderophores. Analytical Biochem. 160: 47-56.

26. Sharma A, Johri B.N (2003). Combat of irondeprivation through a plant growth promoting fluorescent *Pseudomonas* strain GRP3A in mung bean (*Vigna radiata* L. Wilzeck). Microbiol. Res. 158: 77-81.

27. Rodriguez H, Fraga R (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 17(4-5): 319-339.

28. Vivas A, Biro B, Ruı´z-Lozano J.M., Barea J. M.,Azco´n R (2006). Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity Chemosphere. 62: 1523-1533.